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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2023.168.176

Research Article
Biofilm-Forming Heavy Metal Resistance Bacteria From Bungus Ocean Fisheries Port (PPS) West Sumatra as a Waters Bioremediation Agent

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Abstract

Background and Objective: Heavy metals are one of the most worrisome pollutants due to their toxicity. Prolonged exposure to heavy metals and their accumulation and biomagnification properties adversely affect aquatic biota and human health. The ability of microorganisms to bioremediate heavy metals into non-toxic forms is one solution. The research aims of the study were to find biofilm-forming heavy metal-resistant bacteria isolated from the waters of the Bungus Samudra Fishery Port (PPS), Padang City. **Materials and Methods:** This study used a marine agar medium modified with the addition of $K_2Cr_2O_7$, $Pb(NO_3)_2$ and $CdSO_4 \cdot H_2O$, Marine Broth medium and Congo Red Agar medium. The research methods include, the isolation of bacteria, isolate resistance test to heavy metals, testing the ability of isolates to form biofilms and determine the ability of isolates to reduce heavy metals. Furthermore, molecular identification of bacterial isolates was carried out to determine the type of species. **Results:** Five heavy metal-resistant bacterial isolates were found that were able to form biofilms, namely isolates B3Cd, B5Cr, B7Pb, B6Pb and B3Pb. The five isolates were able to reduce heavy metal content by 38.67-61.191%. Identification of the best bacterial isolates on each heavy metal tested, namely B3Cd, B5Cr and B7Pb, respectively, showed the type of *Acinetobacter schindleri*, *Acinetobacter* sp. and *Bacillus* sp. **Conclusion:** These three selected potential isolates can be used as bioremediation agents in metal-polluted waters in the future.

Key words: *Acinetobacter*, bioremediation, cadmium, chromium, lead, pollutant

Citation: Febria, F.A., F. Zulkhairiah, F. Walpajri, A. Putra and L. Syukriani, 2023. Biofilm-forming heavy metal resistance bacteria from Bungus Ocean Fisheries Port (PPS) West Sumatra as a waters bioremediation agent. Pak. J. Biol. Sci., 26: 168-176.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Based on Dewata and Putra[1], the level of pollution in Bungus waters is centred in three locations including, Bungus Ocean Fisheries Port (PPS), Dempo Pertamina and PLTU Teluk Sirih, which showed higher index values than KEPMEN LH No/5/2004 quality standards, namely Cd (0.0013-0.0031 mg L^{-1}), Cr^{+6} (0.008-0.013 mg L^{-1}), Pb (0.009-0.017 mg L^{-1}) and Cu (0.010-0.014 mg L^{-1}). According to the World Health Organization (WHO), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), lead (Pb), nickel (Ni), mercury (Hg) and zinc (Zn) are included in the group metals that are harmful to the environment[2].

Heavy metals are dangerous because they accumulate in the environment and living organisms. Metals are stored more rapidly than they are metabolized or excreted, resulting in an increase in concentration over time. This can disrupt the balance of aquatic ecosystems. Toxicity from heavy metals causes a decrease in mental and central nervous function, lower energy levels and damage to the composition of blood, lungs, kidneys, liver and other vital organs in humans[3]. Various efforts need to be made to restore the environment from environmentally friendly pollutants, one of which is through bioremediation techniques using the services of microbes. Bioremediation is a promising technology for the recovery of heavy metals in the environment, such as in polluted water and soil. Microorganisms carry out detoxification processes such as biosorption, bioaccumulation, biotransformation and biomineralization, which are used for bioremediation both *ex-situ* and *in situ*[4]. Bacteria that are resistant to metals can be isolated from polluted locations and can be used as environmental bioremediation agents. The ability of bacteria to grow and form biofilms as a microbial consortium is a form of defense for survival under unfavorable environmental conditions[5].

Bacterial biofilms it is a group of cells that are organized and agglomerated in a self-produced hydrated matrix known as EPS (exopolysaccharide). Bacterial cells that are protected in this sessile have the potential to protect themselves from various environmental pressures such as changes in pH, salt content and hydraulic pressure. The EPS consists of polysaccharides, proteins, uronic acids, lipids and humic substances. EPS which is poly-anionic forms organo-metal complexes with multivalent cations using electrostatic interactions. More than 90% of metals are remediated by biofilm-forming microbes that have binding ability by EPS[6].

The aim of this study was to find biofilm-forming bacteria that are resistant to heavy metals isolated from Bungus waters of West Sumatra which can later be used as bioremediation agents in metal polluted water.

MATERIALS AND METHODS

Study areas: This research was conducted from April to October, 2022. The research was conducted in the waters of the Bungus Ocean Fishing Port (PPS), Padang City, West Sumatra, Indonesia. Further research is carried out in the Microbiology Laboratory, Central Laboratory and Agro-Technology Laboratory at Andalas University Padang Indonesia.

Sampling: As 1 mL water samples were taken on submerged and textured substrate surfaces such as slime on rocks, wood and shipwrecks. The sample is put into a sterile sample bottle[7]. Then measurements of environmental parameters such as pH, temperature and salinity were carried out.

Making marine agar (MA) medium: As 55.5 g of medium powder dissolved in 1 L of distilled water and then sterilized by autoclaving sea agar[8].

Preparation of medium marine broth (MB): As 40.25 g of Zobell Marine Broth medium powder was dissolved in 1 L of distilled water and then sterilized by autoclaving[8].

Preparation of Congo red agar

Medium: The composition of Congo red agar medium consists of Brain Heart Infusion (BHI) broth (37 mg L^{-1}), sucrose (5 mg L^{-1}), agar (10 mg L^{-1}) and Congo red dye (0.8 mg L^{-1}). Congo red staining was made separately and sterilized by autoclaving at 121°C for 15 min. After that, it was added to BHI broth and sucrose with congo red dye at 55°C[9].

Isolation of heavy metal resistant bacteria: The sample was homogenized using a vortex and then the sample was diluted to 10^{-6}. Isolation was carried out using the pour plate technique using MA medium modified with 10 ppm heavy metals and then incubated for 24 hrs at room temperature. After that, the growing bacterial colonies were observed and purified to obtain pure isolates[10].

Resistance test of bacterial isolates to metal levels in stages: Heavy metal resistance test was carried out by growing bacterial isolates on a modified MA medium with the addition of heavy metal compounds $K_2Cr_2O_7$, $Pb(NO_3)_2$, $CdSO_4 \cdot H_2O$ with concentration levels starting from 50, 100, 200 and 400 ppm. The culture was grown for 24 hrs at room temperature[10].

Testing the ability of bacterial isolates to form biofilms: To find metal-resistant bacterial isolates capable of forming biofilms, they were tested using a Congo red agar medium. Isolates were streaked onto the surface of the medium and incubated at 37°C for 48 hrs. The positive bacteria that form the biofilm colony will turn black-red, while the negative bacteria that form the biofilm are pink to red[7].

Test of isolate ability in reducing metal content quantitatively: Quantitative testing of metal content reduction by selected bacterial isolates was carried out using an MB medium with the addition of metal at a concentration of 100 ppm, then incubated on a shaker (STUART SI50) for 4 weeks. Analysis of metal content was carried out using the Atomic Absorption Spectrophotometry (AAS) technique. Measurement of OD (Optical Density) turbidity using a UV VIS spectrophotometer (EMC-11-UV) at a wavelength of 600 nm was carried out in the Microbiology Laboratory of the Department of Biology Andalas University, Padang.

Molecular identification of bacterial isolates: The best bacterial isolates were carried out by DNA isolation and 16s rRNA gene amplification using primers 27F (5'-AGAGTTTGACA TGGTCAG-3') and 1492R (5'-TACGGCTAC CTTGTTACGA-3') for a total of 35 PCR reaction cycles. Furthermore, the PCR products were sequenced in two directions with the Sanger Sequencing method[11], carried out at the sequencing service provider company, namely 1st Base, Singapore. The sequencing results were followed by Bioinformatics analysis.

Statistical analysis: Statistical analysis using the experimental method with purposive sampling.

RESULTS AND DISCUSSION

Bacterial isolation: Table 1 showed that the highest number of colonies was found in the Marine Agar medium with the addition of $K_2Cr_2O_7$ with a total colony of 4.3×10^4 CFU mL^{-1} and the least number of colonies in Marine Agar with the addition of $Pb(NO_3)_2$ with a total colony of 1.4×10^4 CFU mL^{-1}. The difference in the number of bacterial colonies found indicated that the addition of various metals to the Marine Agar medium affected the number of colonies that were able to grow.

According to Bruins *et al.*[12] the tolerance of bacterial isolates to metals is influenced by several factors including the type of transport of metal ions into cells, the localization of metal resistance genes and the role of these metal ions in cell metabolism processes. In addition, the diversity of bacterial colonies found was also influenced by environmental factors where the samples were taken. The Bungus PPS waters in Padang City have an environmental temperature value of 30.5°C, an alkaline pH of 7.71 and a salinity level of 30 ppt (Table 1). This affects the diversity of microorganisms that live in the environment. In the opinion of Howard[13], environmental physical, chemical and biological factors affect the population size and growth of bacteria. The total number of isolates found was 18 isolates.

The results of testing the resistance of bacterial isolates to metal administration in stages, starting from low concentrations to the highest concentrations of the tested bacterial isolates were able to survive, can be seen in Table 2.

Table 2 shows that the 18 bacterial isolates found from the isolation were able to grow very well (+++) at the administration of the three heavy metals tested up to a metal concentration of 100 ppm, but when the metal concentration was increased to 200 ppm, some isolates grew well (++) and grew less well (+) in the administration of Cd and Cr metals. When the metal concentration was increased to 400 ppm, the growth of isolates was getting worse (+), especially with the addition of Cd metal, even isolates B2Cd and B5Cd did not grow (-). The isolates that were able to withstand the highest metal concentration, namely 400 ppm, in this study, were grouped into the category of metal-resistant bacterial isolates.

The metal toxicity level for all isolates is Cd>Cr>Pb. Slightly different from the study of Gutiérrez-Ravelo *et al.*[14] with the toxicity level of Pb metal being greater than Cd and Cr. This is because bacteria have different abilities in terms of mechanisms of resistance to heavy metals. For example, about Cd metal, bacteria can remove Cd^{2+} ions from their environment by adsorption on the cell wall or accumulation of metal intracellularly. However, this hyperaccumulation of metal ions can interfere with cell physiological processes by forming reactive oxygen species (ROS) production and protein disruption[15].

Cu and Cr metals are metals that are involved in the electron transfer process in bacterial cells and are essential, but when the concentration is excessive, these metals will be toxic to bacteria[16], this can be seen in all isolates tested using Cr metal were able to grow at concentrations of up to 200 ppm, but when it was 400 ppm, the isolates did not grow properly.

Testing the ability of metal-resistant bacterial isolates to form biofilms was carried out using congo red agar medium. Positive bacterial isolates forming biofilm colony color will change from pink to black. The test results of biofilm-forming bacterial isolates were shown in Table 3.

Table 1: Results of bacterial isolation from Bungus Fisheries Port (PPS) waters, Padang City

Source isolate	Number of bacteria (x10⁴ CFU mL⁻¹)	Number of isolates	Environmental factor		
			Temperature (°C)	pH	Salinity (ppt)
Marine Agar+$K_2Cr_2O_7$	4.3	5	30.5	7.71	30
Marine Agar+$Pb(NO_3)_2$	1.4	8			
Marine Agar+$CdSO_4$•H_2O	3.7	5			
Amount		18			

Table 2: Resistance test of bacterial isolates from Bungus Ocean Fisheries Port (PPS) waters against heavy metals at graded concentrations

Isolate code	Concentration of metal addition (ppm)			
	50	100	200	400
B1Cr	+++	+++	++	++
B2Cr	+++	+++	+++	+++
B3Cr	+++	+++	+++	+++
B4Cr	+++	+++	+++	++
B5Cr	+++	+++	++	++
B1Pb	+++	+++	+++	+++
B2Pb	+++	+++	+++	++
B3Pb	+++	+++	+++	+++
B4Pb	+++	+++	+++	++
B5Pb	+++	+++	+++	+++
B6Pb	+++	+++	+++	+++
B7Pb	+++	+++	+++	+++
B8Pb	+++	+++	+++	+++
B1Cd	+++	+++	++	+
B2Cd	+++	+++	++	-
B3Cd	+++	+++	++	++
B4Cd	+++	+++	++	+
B5Cd	+++	+++	+	-

+: Growing less well, ++: Growing well +++: Growing very well and -: Not growing

Table 3: Positive metal-resistant biofilm-forming bacterial isolates

Specific metals	Biofilm-forming positive isolates	Number of isolates
$K_2Cr_2O_7$	 B5Cr	1
$Pb(NO_3)_2$	 B4Pb B6Pb B7Pb	3
$CdSO_4$•H_2O	 B3Cd	1

Table 3 shows that 5 positive metal-resistant bacterial isolates formed biofilms, namely isolates B5Cr, B4Pb, B6Pb, B7Pb and B3Cd. According to Koechler *et al.*[17], the formation of biofilms is regulated by a quorum sensing mechanism, namely communication between cells to form cell groups and provide higher nutrition and increase tolerance to environmental stress. This will protect the bacterial cells until the matrix becomes saturated with metals. The biofilm matrix absorbs metals by a bioabsorption mechanism[16]. According to Salta *et al.*[18], the bacterium *Pseudomonas aeruginosa* with code PAO1 which produces biofilms when treated with Cu metal, dead bacterial cells are found on the outer surface of the biofilm while living cells inside the biofilm are not affected by the metal. An increase in EPS and carbohydrate composition in biofilms occurred when treated with metal stress compared to bacterial cells that were not given stress.

Quantitatively testing the ability of metal-resistant bacterial isolates to form biofilms in reducing metal content was carried out on each of the same media containing different types of metal at a concentration of 100 ppm. The results of the percentage of residual metal content were shown in Table 4.

Table 4 and Fig. 1 show the percentage of residual metal content in isolate B3Cd as much as 41.49%, isolate B5Cr as

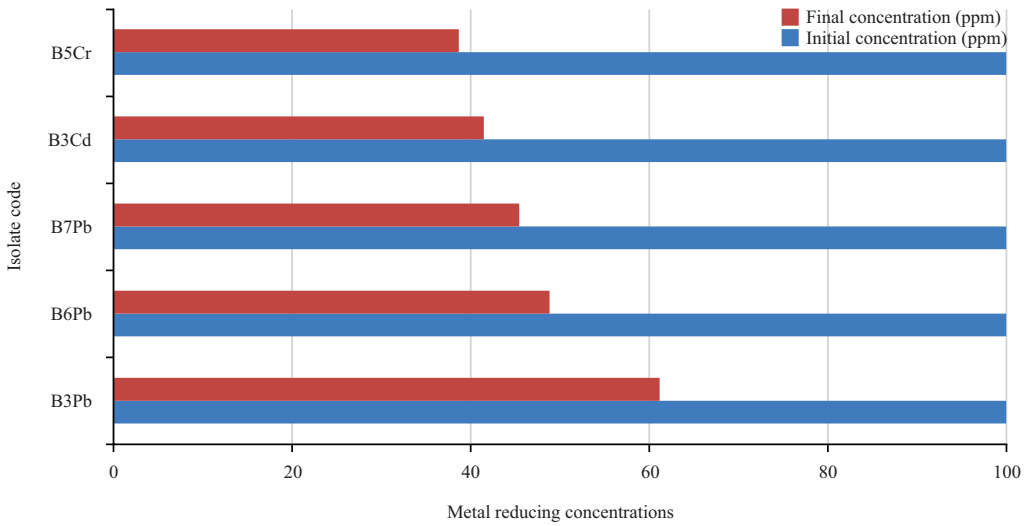

Fig. 1: Percentage of metal reduction by metal-resistant biofilm-forming bacterial isolates analyzed using Atomic Absorption Spectrophotometry (AAS)

Table 4: Percentage of remaining metal content derived by metal-resistant biofilm-forming bacterial isolates

Isolate	Metal	Decrease in metal content (%)
B3Pb	Pb	61.19
B6Pb		48.84
B7Pb		45.46
B3Cd	Cd	41.49
B5Cr	Cr	38.67

much as 38.674% and isolates B7Pb, B6Pb and B3Pb, respectively 45.461, 48.842 and 61.191%. The decrease in the content of each metal indicates the ability of the isolate to handle metals microbiologically. The highest absorption was found in Cr, the highest was in B5Cr isolate and the lowest absorption occurred in B3Pb isolate. This was due to the ability of isolated microorganisms to carry out extracellular detoxification mechanisms that occur. This is due to the interaction of Cr with the hydroxyl groups in the cellulose that coats the bacterial cell wall B5Cr. Absorption of Cr on the bacterial cell wall will prevent Cr from entering the cell or reduce the number of cells that enter the cell[19].

According to Pearson[20], in general, the mechanism for cleaning heavy metals by microorganisms is the ion exchange process. The mechanism of cell metabolism is divided into processes related to metabolism and processes that are not related to cell metabolism, while based on the position of heavy metals can be divided into, extracellular accumulation (precipitation), intracellular accumulation and metal uptake by the cell surface. In the process of metabolism, heavy metals accumulate in the cell membrane (extracellular) and the cytoplasm (intracellular). During the bioremediation process, microorganisms produce enzymes that can modify the structure of toxic pollutants to become less complex so that they become non-toxic and dangerous metabolites. According to Rajendran *et al.*[21] microorganisms in general, microorganisms have a protective mechanism against toxic metals to survive. If the concentration of metals around the environment is high accumulation can inhibit cell growth because the microorganism protection system is no longer capable to compensate for the toxic effects of the metal. The decrease in heavy metal concentrations was also influenced by nutritional factors and an increase in the number of cells in the culture medium.

Identification was carried out on three isolates of bacteria resistant to each metal Pb, Cd and Cr (isolates B7Pb, B5Cr and B3Cd) which were capable of forming biofilms. The results of DNA isolation for each isolate can be seen in Fig. 2.

Figure 2 shows the visualization of bacterial genomic DNA. In isolates B3Cd and B5Cr the intensity of the bands was thicker, while in isolate B7Pb the bands were thinner but still visible. This was due to the low concentration of genomic DNA. Various band intensities indicate differences in DNA concentration. The higher the intensity of the band that appears indicates the higher concentration of DNA and vice versa. Single bands of DNA obtained from each sample also

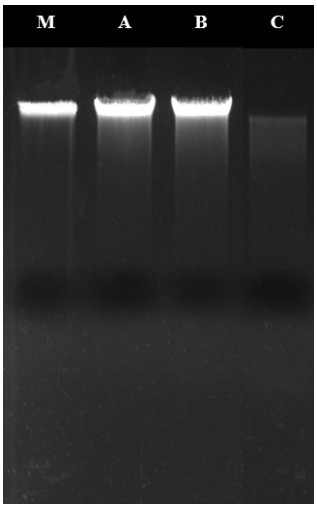

Fig. 2: Visualization of bacterial genome DNA
M: Marker λ DNA (concentration 100 ng μL^{-1}), A: Isolate B3Cd bacterial genomic DNA, B: Bacterial genomic DNA isolate B5Cr and C: Bacterial genomic DNA isolate B7Pb

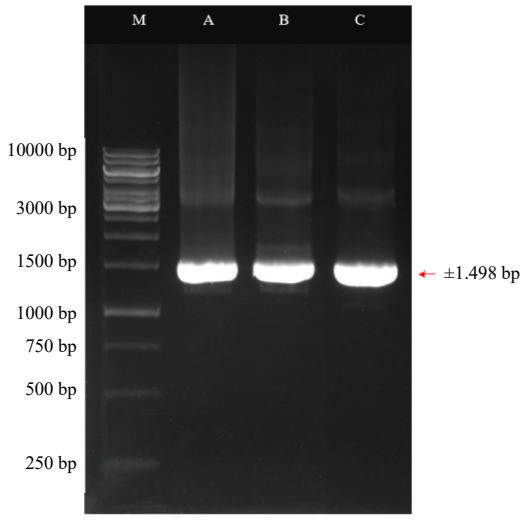

Fig. 3: Results of 16SrRNA gene amplification of bacterial isolate
M: Marker 1kb gene ruler (ThermoScientific, USA), A: 16SrRNA gene PCR product B3Cd isolate, B: Produk PCR gen 16SrRNA isolate B5Cr and C: Produk PCR gen 16SrRNA isolate B7Pb

indicated that there were no contaminants in the form of RNA or protein. According to Pavasant *et al.*[22], electrophoresis can be used to analyze the quality of nucleic acid samples.

Quantification data for bacterial isolates DNA samples were shown in Table 5.

The DNA purity was measured at the ratio of absorbance to wavelength 260/230 nm and 260/280 nm. The value of the absorbance ratio of 260/230 nm ranges from 2.19 to 2.25 (Table 5). If the measurement ratio obtained is lower, it means that the sample is contaminated by EDTA, carbohydrates and phenolic compounds. Based on the data obtained, it has a fairly good level of purity. Bacterial DNA amplification was shown in Fig. 3.

Data from sequencing results were analyzed to determine the kinship of isolates B3Cd, B5Cr and B7Pb using BLAST (Basic Local Alignment Search Tools) from NCBI. The sequence was aligned using Clustal W and the phylogenetic tree construction was performed using the MEGA X application. The phylogenetic tree was constructed using the Neighbor-Joining method with a bootstrap value of 1,000. The evolutionary distance was analyzed using kimura 2-parameter method. Phylogenetic tree showing kinship between bacterial isolates B3Cd, B5Cr and B7Pb with 15 comparison bacteria from genbank based on 16s rRNA gene fragment sequences. the results of the analysis are as follows.

Isolate B3Cd: Based on the construction of the phylogenetic tree, there are two clusters (cluster A and cluster B). Cluster A consisted of 13 bacteria while cluster B consisted of 3 bacteria. B3Cd isolate is in cluster B. In the nearest branch, the B3Cd isolate is in the same branch as the bacterium *Acinetobacter schindleri* strain H3 (CP.030754.1) in Fig. 4. Then in the next branch followed by the *Acinetobacter schindleri* strain ALB6 (MN099373.1). The position on the branch of the phylogenetic tree between B3Cd isolate and these 2 bacteria show the closest degree of kinship, with a percent identity of 99.32-99.77% with a genetic distance value of 0.002, then the B3Cd isolate is *Acinetobacter schindleri*.

Isolate B5Cr: Based on the construction of the phylogenetic tree, there are two clusters named cluster A and cluster B. Cluster A consists of 13 bacteria while Cluster B consists of 3 bacteria. Cluster A is further divided into sub-clusters A.1 (7 bacteria) and A.2 (6 bacteria). Then sub-cluster A.1 is divided into sub-clusters A.1.a (5 bacteria) and A.1.b (2 bacteria). Isolate B5Cr is found in sub-cluster A.1.a. Members of sub-sub-cluster A.1.a apart from isolate B5Cr a are *Acinetobacter schindleri* strain ACE, *Acinetobacter schindleri* strain H4-3-C1, *Acinetobacter* sp., fl9(2011) and *Acinetobacter* sp., Cai-20. If seen from the BLAST results in Fig. 5. The construction of the phylogenetic tree, the percent identity obtained is 99.27-99.64% and the genetic distance value is 0.003, then the B5Cr isolate is the bacterium *Acinetobacter* sp.

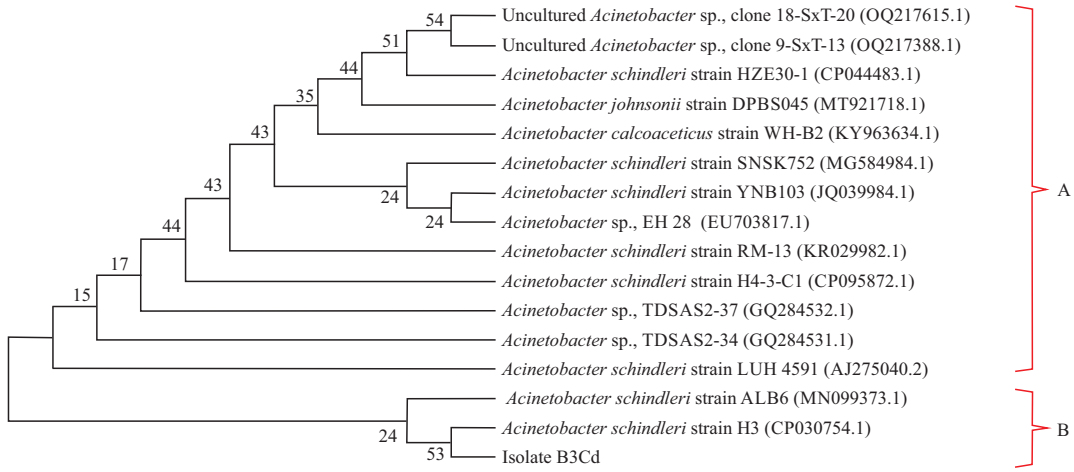

Fig. 4: BLAST results and phylogenetic construction of the B3Cd isolate

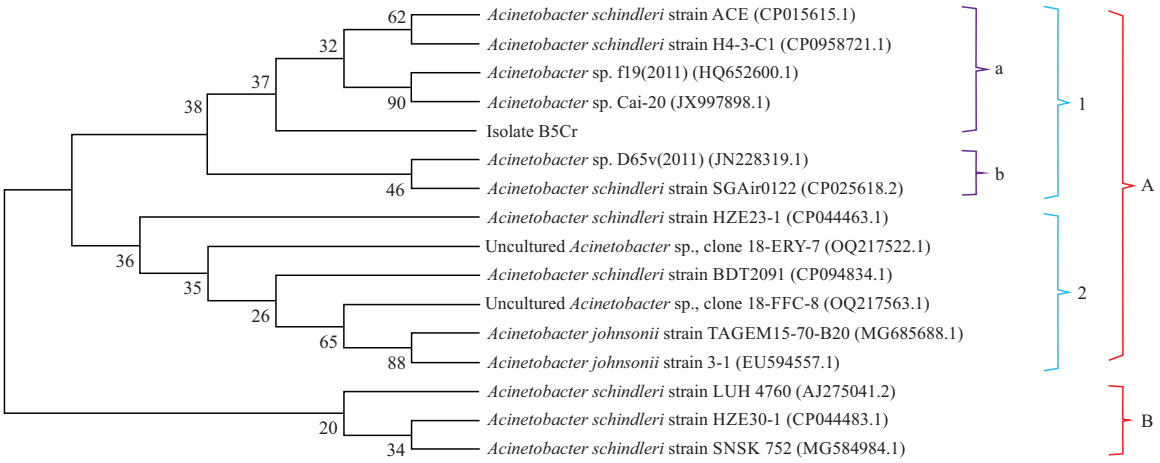

Fig. 5: BLAST results and phylogenetic construction of B5Cr isolate

Table 5: Quantification data of bacterial genomic DNA samples

Bacterial isolates code	Concentration (ng μL^{-1})	Absorbance of A260/A230	Absorbance of A260/A280
Cd Isolate	124	2.21	1.98
Cr isolate	137	2.19	1.98
Pb Isolate	12	2.25	1.70

B7Pb isolate: Based on the construction of the phylogenetic tree, there are no clusters that differentiate the kinship groups between the bacteria being compared. Isolate B7Pb with 15 comparators from the genbank were both on a single branch with a value of 100 can see in Fig. 6. If looking at the BLAST results, alignment with Clustal W and calculation of genetic distance, it was found that there were no differences between the bacteria. Therefore, isolate B7Pb is *Bacillus* sp., 16S rRNA gene sequence cannot discriminate between bacterial species in the genus *Bacillus* sp., developed a new method for molecular identification of bacteria using housekeeping genes, namely the dnaJ, dnaK, mutL, pheS and yycH genes.

The implication of this research is that it can provide a way to overcome the problem of heavy metals that pollute the environment by using bacteria obtained from the source of the heavy metal contamination, the application of this research helps in reducing heavy metal contamination that pollutes the environment with the help of bacteria that have been identified so that these bacteria can be cultured in large quantities which will be ready to be released in polluted environments, the recommendations in this study are that it

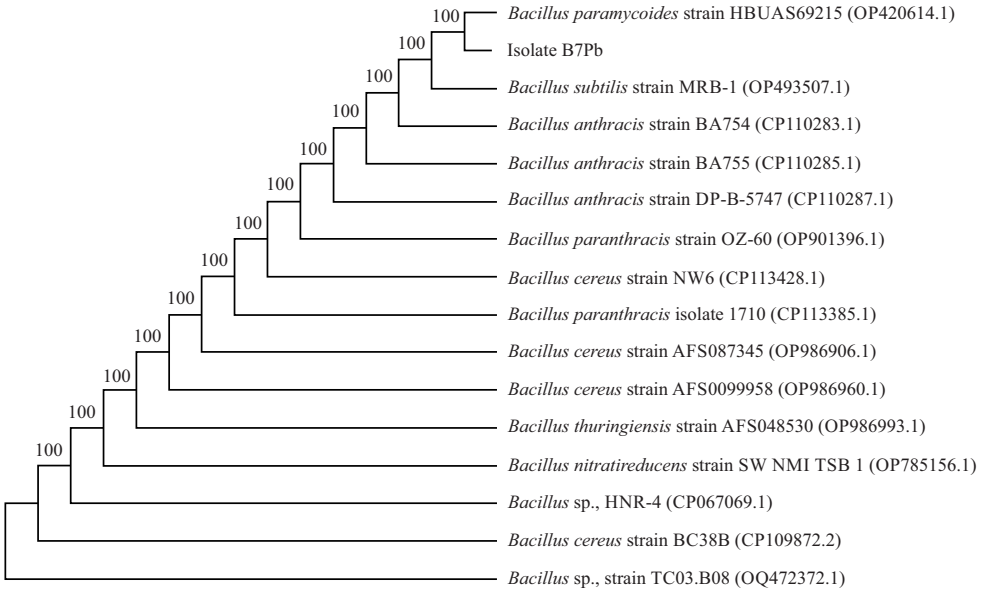

Fig. 6: BLAST results and phylogenetic construction of isolate B7Pb

is hoped that *in vitro* experiments and optimization of bacteria obtained with heavy metals and the limitations of this research in testing metal reduction requires a long time.

CONCLUSION

Found 5 isolates of bacteria that have the ability can form biofilms, 1 isolate of which is resistant to metal Cd, 1 isolate is resistant to metal Cr and 3 isolates are resistant to metal Pb. The three isolates have been tested to reduce metal content up to 38.67-48.84%. Molecular identification of the three selected potential isolates for each metal indicated B3Cd isolate is *Acinetobacter schindleri*, isolate B5Cr is *Acinetobacter* sp. and Isolate B7Pb is *Bacillus* sp. The three selected potential isolates can be used as bioremediation agents in metal-polluted waters in the future.

SIGNIFICANCE STATEMENT

This research was conducted because heavy metal pollution in the sea is increasing due to pollution from ships and factories, therefore it is necessary to search for candidate biofilm bacteria around polluted seas to find candidate bacteria capable of remediating pollutants in the form of heavy metals. The study was to find biofilm-forming heavy metal-resistant bacteria isolated and obtain a type of bacteria that has the potential to reduce heavy metal contamination, this is important because in the future it can be developed and cultured more for more in-depth experiments and applications in polluted environments. The next study is necessary to optimize and experiment *in vitro* with these bacteria so that they can be used in direct application to the field.

ACKNOWLEDGMENTS

The author would like to thank DIKTI for allowing us to carry out this research by agreeing to fund this research. This research was funded by Andalas University PNBP for Fiscal Year 2022, with contract number T/7/UN.16.17/PT.01.03/IS-RD/2022.

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